GenVinSet

HFE-HEMOCHROMATOSIS

Instructions for Use

Kit for the detection of the C282Y mutation of the Hemocromatosis gene (HFE)

Reference GVS-C282Y-48 (48 test)
GVS-C282Y-24 (24 test)

Store from –18 to –30ºC

For In Vitro Diagnostic Use CE

Rv 03/10-10-2017

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Intended use

GENVINSET HFE C282Y is a kit for the determination of the C282Y mutation, associated with primary hemochromatosis, by real time PCR using Taqman® probes technology.

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Summary and explanation

Hereditary hemochromatosis (HH) is an autosomal recessive inherited disorder of iron metabolism. Due to excessive intestinal absorption, iron accumulates in the parenchymal cells of the liver, pancreas, heart and other organs resulting in damage to its structure and its function impaired. It is one of the most common genetic diseases in Caucasians with a prevalence of about 1 in 300 [1]. Although the disease symptoms are often non-specific, much of the organ damage is irreversible once it has occurred. Early detection and treatment is therefore very important as part of preventive medicine. The discovery of the responsible gene called HFE in 1996 resulted in the inclusion of molecular analysis in the diagnostic strategy for HH [2].

The HFE gene is located on the short arm of chromosome 6, it encodes a 343 amino acid glycoprotein called HFE protein, which has similarities with proteins of the major histocompatibility complex class I [1]. A number of different HFE mutations have been described. Most HH cases (52-96%) in the European regions are associated with a homozygous mutation at position 845 (G —› A) of exon 4 of the HFE gene, which results in an amino acid change at position 282 from cysteine to tyrosine (C282Y) [1,2]. There is a second mutant allele at position 187 (C —› G), detected with a relatively high frequency in exon 2 of the HFE gene wherein the amino acid histidine is replaced by an aspartic acid at position 63 (H63D) [2]. The contribution of this allele for iron overload is most relevant in the case of heterozygosity combined with allele C282Y (C282Y/H63D) [3,4]. The third HFE mutation is a substitution at position 193 (A —› T) of exon 2 resulting in an amino acid change at position 65 from serine to cysteine (S65C) and has proven to be generally benign, although the C282Y / S65C genotype can impart a slight increase in disease risk, contributing to a mild disease phenotype [4-6]. Many of the other HFE mutations described are private and rare, or found only in certain regions [7].

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Procedure principles

The detection method used by GENVINSET HFE C282Y is based on a primer specific PCR, which anneals to exon 4 of the HFE gene and two specific Taqman® probes for normal and mutated genes.

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Kit contents

Reference GVS- C282Y -48 (48 tests)

- GVS-C282Y-PM: 2 vials x 110 µL Primer Mix (PM)
- GVS-HFE-MM: 2 vials x 156 µL Master Mix (MM)
- GVS-C282Y-C1: 1 vial x 5 uL Control WT (C1)
- GVS-C282Y-C2: 1 vial x 5 uL Control MUT (C2)
- GVS-RB: 1 vial x 100 uL Reaction Blank (RB)

Reference GVS- C282Y -24 (24 tests)

- GVS- C282Y -PM: 1 vial x 110 µL Primer Mix (PM)
- GVS-HFE-MM: 1 vial x 156 µL Master Mix (MM)
- GVS-C282Y-C1: 1 vial x 5 uL Control WT (C1)
- GVS-C282Y-C2: 1 vial x 5 uL Control MUT (C2)
- GVS-RB: 1 vial x 100 uL Reaction Blank (RB)

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Kit storage

All of the kit's reagents should be stored from -18ºC to -30ºC, they are stable at this temperature until the expiration date, as indicated on the bottle. Do not perform more than 3 freezing/thawing cycles to the Primer Mix (GVS-C282Y-PM) and Master Mix (GVS-HFE-MM) vials as this could reduce the assays sensitivity and change results.

Due to the reagents' photo sensitive nature, avoid continuous exposure to light.

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Materials required but not supplied

General

- Gloves
- Lab coat

Consumables

- Filter tips (P1000, P200 & P20)
- 1.5 mL autoclaved tubes
- RT-PCR instrument specific reagents (in the case of using Rotor-Gene Q, only 0.1mL tubes are allowed)

Equipment

- Real time PCR instrument
- Vortex
- Pipettes (P1000, P200 & P20)

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Sample collection and preparation

This test should only be performed with complete blood samples treated with EDTA anti-coagulation agents or citrate. Heparin can interfere with the PCR process and should not be used in this procedure.

It is recommended to test the DNA extraction system with GENVINSET HFE C282Y before using the results for diagnostic purposes.

Caution

All biological and blood samples should be treated as possibly infectious. When manipulating them, observe all basic (universal) precautions. All sample manipulation should be done with gloves and appropriate protection.

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Procedures

PCR preparation

Precautions

- Thaw all of the kits components before starting the assay, mix and centrifuge.
- Work over ice or a cold block.
- The PCR should be assembled in the Pre-PCR area.
- Use only filtered tips and 1.5mL autoclaved tubes.
- Use gloves and a laboratory coat at all times.
- In each session it is recommended to use one negative sample for C282Y mutation, one homozygous sample for C282Y mutation and one heterozygous sample.

1. Prepare the Primer Mix and Master Mix mixture for n+1 samples:

	Vol. per sample (µL)
Primer Mix (PM)	4
Master Mix (MM)	5

2. Pipette 9 µL of this mixture into the optical plate and add 1 µL of the DNA sample or negative control (in the case of contamination control well).

3. Seal the plate with convenient sealer, spin down the volume by centrifuging for 1 minute at 360 x g.

4. Place the plate in the thermal cycler and start the amplification program described below.

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Thermal cycler configuration

1. Set up the next amplification program:

	Cycle Number	Temperature (ºC)	Time (mm:ss)	Ramp (%)	Analysis
Denaturation	1	95	05:00	100	X
Cycles	50	95	00:15	100	X
		64	1:00	100	Single
Cooling	1	15	∞	100	X

2. Set up the reading channels.

The emitted fluorescence must be read in FAM (495-520 nm) and HEX (535-554 nm) channels. Both fluorescences should be detected in every well (biplex reaction).

NOTE – Special settings for Rotor Gene Q:

a. Open the Rotor-Gene Q – Pure Detection software. Select the tab "Advanced" in the window New Run, and click "New".

b. Select the type of rotor used (only 0.1 mL tubes accepted, see section 'Materials required but not supplied', page 8). Select the "Locking Ring Attached" box and continue by clicking "Next".

c. Type the "Reaction Volume" as 10 µL, and identify the operator and the sample.

d. Click "Edit Profile" and set up the amplification program (see subsection 'B)Thermal cycler configuration'). Select the step 60 sec at 60 ºC, and click "Acquiring to Cycling A". Select the channels for fluorescence acquisition "Green" and "Yellow". Then "OK". Click "OK" to accept and close the"Edit Profile" window.

e. Clic "Gain Optimisation" in the "Run New Wizard" dialog box to open the "Auto-Gain Optimization Setup" window. In the scroll menu of "Channel Settings" select "Acquiring Channels" and then "Add". In the window "Auto-Gain Optimisation Channel Settings", set the following parameters for each channel ("Green" and "Yellow"):

- Tube position = 1
- Target Sample Range: 5 FI up to 10 FI
- Acceptable Gain Range: -10 to 10

f. Activate the option "Perform Optimisation Before 1st Acquisition", and click "Close".

g. Select "Next" and then "Start Run" in the "New Run Wizard" window.

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Results

GENVINSET HFE C282Y is a qualitative technique to identify the presence of the normal and/or mutated C282Y allele.

It is not necessary to select any passive reference.

The results of this technique can be obtained as follows:

Amplification curves – Quantitative Experiment

Select the channel FAM to be able to see the amplification curves of the mutated alleles. Select the channel HEX (VIC) to observe normal or wildtype alleles. The presence of an amplification curve in any of the channels mentioned indicates the presence of the corresponding allele (at least one copy).

Figure 1 shows the amplification plot of an assay run. Red curves correspond to the FAM channel, and therefore, the mutated alleles. The green curves correspond to the HEX channel in which the normal alleles are detected.

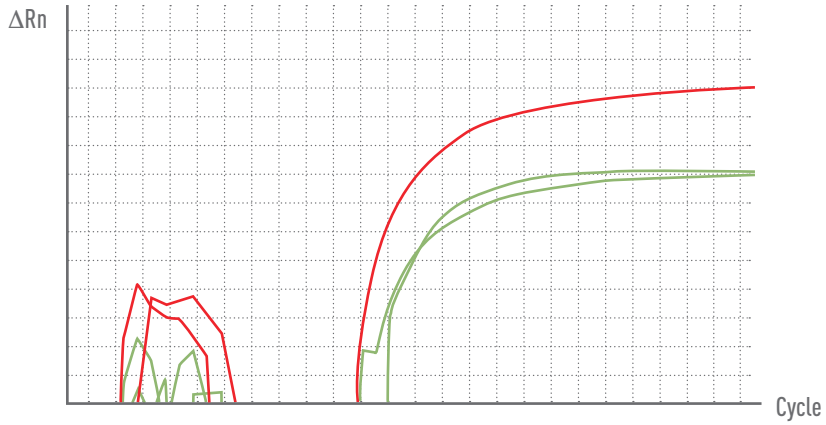

Figure 1. Plot showing the results of one heterozygous sample (wt/mut) and one normal sample (wt/wt) using Genvinset HFE C282Y kit. Amplification signal from HEX in green and from FAM in red.

Genotyping analysis

In Genotyping or Allelic Discrimination analysis types, select FAM channel (mutation) in Y axis, and HEX channel (wildtype) in X axis. The results will be shown similarly to those in Figure 2, as dots with different X and Y components. The different kind of samples, wt/wt, wt/mut and mut/mut, will be distributed into 3 groups within the plot. The Reaction Blank will be placed next to the origin of coordinates (0,0).

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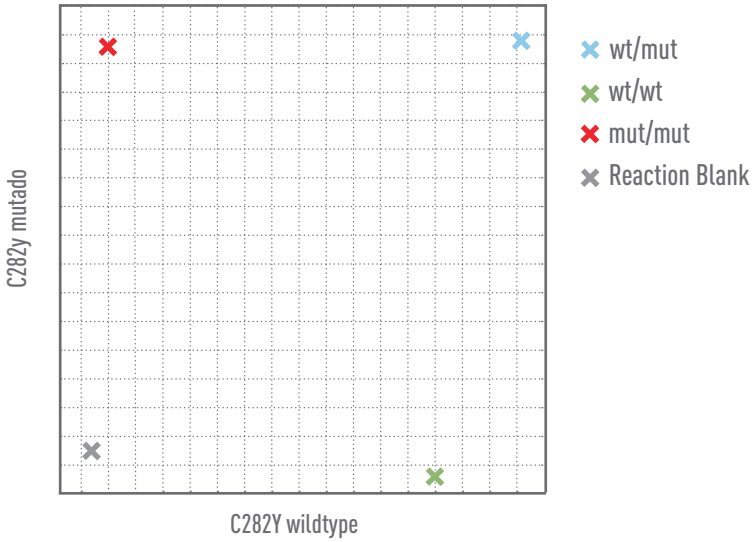

Figure 2. Plot showing one heterozygous sample (wt/mut), one normal sample (wt/wt) and one homozygous mutated sample (mut/mut) for C282Y, using Genvinset HFE C282Y kit. Graphic distribution according to detected alleles

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Quality control

Due to the qualitative nature of this test, it will not be necessary to perform a calibration.

It is recommended to carry out a contamination control by replacing the DNA sample for the Reaction Blank provided with the kit, and to introduce control samples (normal, homozygous and heterozygous).

The following criteria should be known for the assay to be considered valid:

- Reaction Blank must provide negative results for both normal and mutated alleles, negative results considered as those with crossing point (Cp) > 35. Cp values <35 inform about a contamination so the session should be discarded.
- A normal control sample should be positive just for HEX-wildtype channel. A homozygous mutated sample should be positive just for FAM-mutation channel and a heterozygous sample should be positive for both HEX and FAM channels.
- DNA samples generating results with Cp >35 should be considered as doubtful and must be retested by performing a new DNA extraction.

The assay must be made according to the kits recommendations, as well as other quality control procedures that comply with local, federal and/or certifying agencies specifications.

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Specific operation data

1. Analytical specificity

The alignment of primers and probes has revealed the absence of non-specific bindings. No cross-reaction phenomena with genomic DNA have been reported.

2. Analytical sensitivity

A dilution assay was performed using 1:4 serialized dilutions of two DNA samples, one homozygous for C282Y and one heterozygous for C282Y, obtained by a conventional extraction system, at a concentration of 33,40 and 29,88 ng/µL respectively. The following results were obtained for analytical sensitivity of the wildtype/mutated alleles detection:

- DNA obtained by conventional extraction: Detection limit=0,52 ng/µL (*)

(*) Cp < 35

3. Diagnostic sensitivity and specificity

In three studies of human genomic DNA, 96 samples obtained from three clinical laboratories were analyzed. They were previously genotyped by different methodologies.

Out of the 97 samples tested, all of them were validated (positive amplification of at least one allele), except for one in which every amplification failed. The results are the following:

	GENVINSET HFE C282Y		
PREVIOUS METHOD			
Samples	wt/wt	wt/mut	mut/mut
wt/wt	53	0	0
wt/mut	0	34	0
mut/mut	0	0	9

There is a 100% match in the results obtained with GENVINSET HFE C282Y and the genotyping previously obtained with the routine method used in each laboratory.

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Troubleshooting guide

Reaction Blank (H2O) is positive

- **Primer Mix/Reaction Blank contamination**
  - Repeat the experiment with new Primer Mix/Reaction Blank aliquots
  - Perform the kit components manipulation always according to usually accepted practices to avoid contamination
  - Verify manipulation and storage conditions
  - Discard contaminated reagents
- **Pre-PCR area is contaminated**
  - Confirm that all necessary precautions in the PCR area have been followed
  - Check for possible contamination problems in other PCR techniques
  - Confirm suitability of the used reagents (1.5 mL tubes, pipette tips)
  - Confirm there is no Taq contamination
- **Pipetting error**
  - Always check that the added sample matches the sample sheet

Low or no signal in all samples. Control samples are OK.

- **Bad quality of DNA**
  - Repeat the sample extraction verifying each step (Hemoglobin can interfere with the PCR)
- **Samples with very low DNA concentration**
  - Check the DNA concentration and repeat extraction if necessary
- **DNA samples with high concentration**
  - Perform a DNA extraction method validation assay testing some dilution of the DNA samples

Fluorescence intensity too low

- **Kit degradation (Primer Mix vial)**
  - Confirm the kits correct storage (Primer Mix vial stored in darkness)
  - Avoid more than 3 freeze/thaw cycles of the primer mix vial

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- Aliquot the reagents if necessary
- Repeat the series with new reagents

- **Taq has lost activity**
  - Confirm Taq's activity
  - Repeat with new Taq

C1 control sample (C282Y wildtype) is positive

- **Cross contamination**
  - Always handle the kit's components with all current contamination avoidance practices
- **Pipetting error**
  - Always check that the added sample matches the sample sheet

C2 control sample (C282Y mutated) is negative

- **Pipetting error**
  - Always check that the added sample matches the sample sheet

Fluorescence intensity varies

- **There is dirt on the outside which interferes with the signal**
  - Always wear gloves when manipulating the plates/wells
- **The volume is not at the bottom of the well or there is an air bubble**
  - Centrifuge to make sure the sample is at the bottom of the well and there are no air bubbles, according to the technical protocol
- **Pipetting error**
  - Verify the volume added in each well is correct

There is no fluorescence signal

- **Incorrect reading channels selected**
  - Configure the correct reading channels
- **Pipetting error or reagent absence**
  - Control the pipetting and the reactions configuration
  - Repeat the PCR
- **No reading channel was selected in the thermal cycler's program**
  - Revise and modify the thermal cycler program

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